The association and partial characterisation of a fluorescent hypersensitive response of potato roots to the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*

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SUMMARY

The early parasitic development of second stage juveniles of *Globodera* spp. on potato has been studied *in situ* using a novel plant growing technique. This technique has been used with fluorescence microscopy to define the chronology and location of a hypersensitive reaction (HR) in susceptible and resistant cultivars to invasion by *G. rostochiensis* (Ro 1) and *G. pallida* (Pa 2/3). Fluorescence at the point of entry of the nematode and along the track taken towards a feeding site was observed within 3 h of invasion. Fluorescence was a better indicator of the extent of the HR than necrotic browning and was used to measure the distance nematodes travelled within the roots. The extent of fluorescence which resulted from intracellular movement of the nematodes was correlated with the distance they travelled within the roots. Differences in quantified fluorescence among the host-parasite combinations may be correlated with the degree of compatibility of the relationships. Fluorescence was observed in cells surrounding the developing syncytium in incompatible cultivars. Preliminary chemical and spectral analysis of the fluorescent tissues indicated that the fluorescence was due to the accumulation of phenylpropanoid compounds and the involvement of these in the expression of nematode resistance in potatoes is discussed.

RÉSUMÉ

Association d'une réaction d'hypersensibilité en fluorescence des racines de pommes de terre aux nématodes à kyste des pommes de terre Globodera rostochiensis *et* G. pallida, *et sa caractérisation partielle*

Les premières étapes de l'action parasitaire des juvéniles de second stade de *Globodera* spp. ont été étudiées, sur pommes de terre, en utilisant une technique de culture originale. Cette technique a été couplée à l'utilisation de la microscopie en fluorescence pour définir la chronologie et la localisation des réactions d'hypersensibilité (RH) dans des cultivars résistants et sensibles à l'infestation par *G. rostochiensis* (Ro 1) et *G. pallida* (Pa 2/3). Une zone fluorescente a été observée au point d'entrée du nématode et le long de son trajet vers un site nutritionnel, et ce dans les trois heures suivant la pénétration. La fluorescence, meilleur indicateur de l'étendue de la RH que le brunissement nécrotique, a été utilisée pour mesurer la distance parcourue par les nématodes dans les racines. L'étendue des zones fluorescentes induites par les mouvements des nématodes à l'intérieur des cellules est en corrélation avec la distance qu'ils parcourent à l'intérieur des racines. Les variations quantitatives de cette fluorescence suivant les couples hôte/parasite pourraient être en relation avec le degré de compatibilité du couple ou faire partie d'une réponse générale au traumatisme, laquelle varie suivant le cultivar de pommes de terre. Chez les cultivars non compatibles, des zones de fluorescence ont été observées dans les cellules entourant le syncitium en formation. Les premières analyses chimiques et spectrales des tissus fluorescents indiquent que cette fluorescence est due à l'accumulation de composés phénylpropanoïdes; le rôle de ces derniers dans l'expression de la résistance des pommes de terre aux nématodes est discuté.

The incompatibility of plants to certain endoparasitic nematodes has been studied using a wide variety of histological, histochemical, biochemical and ultrastructural techniques. Kaplan and Keen (1980) suggested that the major incompatibility mechanism involves post-infectional hypersensitive reactions (HR). In potatoes, the HR to the potato cyst nematodes (*Globodera* spp. or PCN) involves localised host cell necrosis and browning (Giebel, Krenz & Wilski, 1970; Hoopes, Anderson & Mai, 1978), followed by the disorganisation and lysis of the syncytium induced by the nematode (Rice, 1983) which is a feeding site facilitating transfer of nutrients from plant to nematode (Jones & Northcote, 1972). There are also concomitant or subsequent changes in simple phenols or their oxidation products (Giebel, 1970, 1974). The possibility that post-infectional induction of chemical compounds may modify the compatibility of the host plant to the parasite has been discussed by these workers and others (Veech, 1982). Kaplan and Keen (1980) and Veech (1982) have

stressed in particular the importance of defining the chronology and precise location of chemical changes in the plant following nematode invasion.

Previous research into the response of incompatible plants to fungal invasion has employed techniques based on fluorescence microscopy to examine hypersensitive reactions. The localisation of the highly fluorescent broad bean phytoalexins wyerone and wyerone acid was established by fluorescence microscopy of leaf epidermal tissue (Mansfield, Hargreaves & Boyle, 1974). Mayama and Shishiyama (1978) demonstrated that autofluorescent and UV-absorbing substances accumulating at penetration sites in barley leaves infected with *Erysiphe graminis hordei* could be polyphenol compounds. The main restriction of using fluorescence microscopy to study whole roots infected by PCN is that these tissues are usually too thick and opaque to allow direct observation of invading nematodes. Studies have so far only been made on processed and sectioned roots (Ahmad & Chen, 1983; Arya & Tiagi, 1985). We present in this paper a technique for studying *in situ* the early parasitic development of *Globodera* spp. in the potato. This has been used with fluorescence microscopy to define the chronology and location of changes in fluorescence of the roots of a limited range of clones including both susceptible and resistant responses to both *G. rostochiensis* (pathotype Ro 1) and *G. pallida* (pathotype Pa 2/3).

Materials and methods

Cysts of *Globodera rostochiensis* (Ro 1) and *G. pallida* (Pa 2/3) reared in 1983 were soaked for one week in distilled water prior to stimulation of hatching using potato root diffusate; only nematodes which hatched over the first five days were used for experimentation. The following potato clones and cultivars were used for experimentation : *Solanum vernei* hybrid clone 12380/2, cv. Maris Piper and the susceptible cv. Arran Banner. The *S. vernei* clone was supplied by the Scottish Crop Research Institute and in initial screening, this clone was resistant to *G. pallida* (1-2 % of the reproduction achieved on susceptible cv. Arran Banner) and partially resistant to *G. rostochiensis* Ro 1 (suggesting presence of gene H_1). Maris Piper is resistant to *G. rostochiensis* and contains gene H_1 only.

Sprouted tuber pieces, excised with a cork borer, were planted in sterile sand (150-400 µm particle diameter; BDH Chemicals Ltd) in a central portion of the paper trough of seed growth pouches (Northrup King & Co, 13410 Research Road, Eden Prairie, Minnesota, USA) maintained in a glasshouse at 18-22°. Developing roots grew in the pouches (Fig. 1 A) and when 10-15 cm long, the whole plantlet was removed, washed and transferred to a growth unit (Fig. 1 B) comprising two 9 cm plastic Petri dishes joined together with tape. The roots of the potato plantlet were placed onto a preformed slab of sterile, 2 % water agar in one of the Petri dishes with the growing shoot occupying the adjoining dish; all procedures were carried out in a laminar flow cabinet. The unit was sealed to keep in moisture and kept under the same conditions as the plants in growth pouches. Once secondary roots had developed, up to 100 second stage juveniles were pipetted in 2-5 µl distilled water onto the agar close to the roots. The roots, which were slender and transparent, were then examined with a binocular microscope to assess infection and to record the time and point of invasion. At various intervals following invasion, infected roots were excised and transferred onto a thin (0.5-1.0 mm) layer of agar sandwiched between two coverslips. This arrangement protected the excised roots from desiccation and from being crushed and allowed the root piece to be viewed from either of two sides depending on the location of the nematode.

The prepared root segments were examined under an Olympus incident light fluorescence microscope fitted with a broad-band blue excitation filter (455-500 nm) and 515 nm barrier filter. The distance nematodes moved within the roots could be measured as a result of intense fluorescence associated with the intracellular track of the animal. The intensity of fluorescence associated with infection was quantified over a defined area of the track with a Zeiss photomicroscope fitted with a photomultiplier tube interfaced via a photometer to a microcomputer. Emission spectra of fluorescence were measured using a microspectrophotometer (Leitz MPV 2). The relationship between fluorescence and the onset of browning was analyzed by using scanning densitometry of developed colour transparency film (Kodak Ektachrome 200) of infected roots.

Chemical analysis of fluorescent tissues was done on segments of infected and uninfected roots (1-5 mg wet weight) which were homogenised in 80 % methanol and centrifuged at 10 000 g for 5 min. Nematodes in infected roots were not removed prior to this procedure because it was considered that the autofluorescence of the animal was insignificant compared to fluorescence in damaged tissue. The supernatents were dried under reduced pressure at 35° and made up to standard volume in either 80 % methanol or hydrolysed for 0.5 h in 2M HCl (Harborne, 1984). After hydrolysis and extraction into ether, the extracts were concentrated to dryness and redissolved in 80 % methanol. All extracts were then analysed by thin layer chromatography (t.l.c.) on 10 × 10 cm aluminium-backed plates coated with 0.2 mm thick layers of silica gel 60 GF 254 (Merck). Plates were developed in butan-1-ol, acetic acid, water (6/1/2, v/v/v). Fluorescent spots were detected under UV light (360 nm) and their Rf values compared to co-chromatographed commercial standards of scopoletin, caffeic acid, and chlorogenic acid (SIGMA Chemical Co.) made up in 80 % methanol.

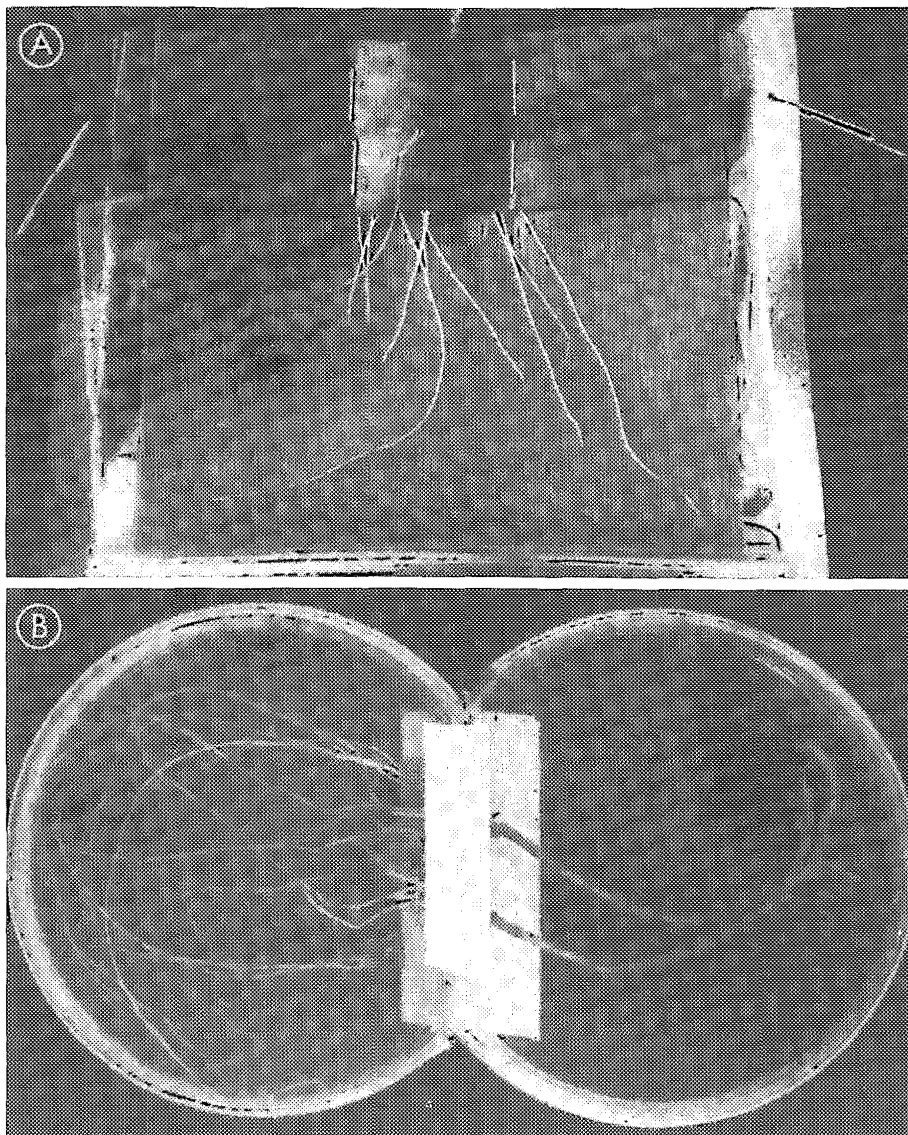

Fig. 1. A : Plastic growth pouch with primary roots of potato sprout growing through perforations in the fold (× 0.7); B : Petri dish growth unit used for development of slender, transparent secondary roots on agar (× 0.6).

Results

Invasion of secondary roots had usually taken place within 48 h of inoculation, and the first signs of fluorescence were seen after 3 h. By 24 h after invasion the area of entry and the track taken by the nematode within the root was clearly defined by the fluorescence associated with the plant response. Intensity was greatest along the cell walls and at the anterior end of the nematode (Fig. 2 A, B). By 24 h most juveniles had become sedentary and presumably begun to initiate a syncytium.

The distance travelled by nematodes within the root was measured after four days (Tab. 1). Analysis of variance indicated that *G. pallida* travelled significantly further than *G. rostochiensis* in Arran Banner and Maris Piper ($P < 0.05$). The shortest distance travelled by both species was in the *S. vernei* hybrid 12380/2. The majority of juveniles moved away from the root tip in Maris Piper and Arran Banner (89 %) whereas in the *S. vernei* hybrid, a large proportion (46 %) travelled towards the root tip causing an intensely fluorescent response (Fig. 2 D).

The microphotometric system was used to measure the relative intensity of the fluorescent response over an

Table 1
Extent of fluorescence and distance travelled by nematodes in potato roots four days after invasion by *Globodera rostochiensis* or *G. pallida.*

		Track fluorescence	*Distance travelled (mm)*
Arran Banner			
G. rostochiensis	C[1]	27.2 ± 4.4[2]	1.15 ± 0.13
G. pallida	C	24.1 ± 4.3	1.6 ± 0.17
Maris Piper			
G. rostochiensis	I	31.1 ± 3.2	1.12 ± 0.20
G. pallida	C	23.6 ± 2.6	2.01 ± 0.18
12380/2			
G. rostochiensis	I	35.5 ± 8.4	0.87 ± 0.07
G. pallida	I	32.9 ± 5.9	0.92 ± 0.09

(1) C = compatible interaction; I = incompatible interaction.
(2) Fluorescence, measured in photometer units, corrected for background autofluorescence. (Mean ± standard error of a minimum of 10 readings.)

area 0.125 mm in diameter along the track taken towards the feeding site (Tab. 1). No assessments were made in the case of multiple invasion. Analysis of eighteen samples revealed that the fluorescence along the track taken by *G. rostochiensis* was significantly greater than that of *G. pallida* in Maris Piper and 12380/2 ($P < 0.05$). There was no difference between the two species on the susceptible cultivar Arran Banner. This indicates that the extent of fluorescence may be related to the compatibility of the host parasite relationship.

The emission spectra from these cells are shown in Fig. 1 along with spectra of certain phenolic standards which may correspond to the fluorescent material. Damaged cells had fluorescent peaks at 540 and 655 nm. The peak at 655 nm was associated with an orange/yellow fluorescence characteristic of the early stages of infection and was not present at 96 h. Undamaged cells and root tips autofluoresced with a peak at approximately 545 nm with less than one sixth the intensity of damaged cells. The prepared standards fluoresced with emission maxima within the range of the main peak associated with nematode damage. The 539 nm peak of caffeic acid corresponded most closely to the main emission peak for infected roots. However, the possibility of quenching of particular wavelengths by plant cell components cannot be discounted and this limits valuable comparison of invasion peaks.

The ratio of browning cells to fluorescing cells, as determined by scanning densitometry, was 16 % after 24 h and increased to 74 % 96 h after invasion. Browning was most intense in cells immediately adjacent to the nematode and appeared fastest in areas of the root around the point of entry.

Fluorescence associated with the developing syncytium was observed four days after invasion. By this time fluorescence in cells surrounding the initial syncytium was well developed whereas within the syncytium fluorescence was less intense. This effect was seen only in incompatible plant-nematode combinations (Fig. 3 B, C).

Preliminary tests of thin layer chromatography of infected and uninfected roots are shown in Table 2. Also shown are the Rf (× 100) values of phenolic standards. The main fluorescent spot of unhydrolysed methanolic extracts of infected roots was at 25.4. This was present in uninfected root extracts but was less intense. These spots had a similar Rf value and colours under UV light to that of co-chromatographed chlorogenic acid. The spot at 83.1 in infected root extracts and 93.0 in both extracts was not identified. The latter may be due to bright autofluorescence in actively growing tips of infected and uninfected roots.

Hydrolysed extracts of infected roots gave two spots at Rf 86.4 and 95.5. The former was the larger of the two. This could correspond to the caffeic acid spot at 88.1 which is the hydrolysis product of chlorogenic acid. Uninfected root extracts produced only one spot at 95.5 which may also be due to root tip autofluorescence. The scopoletin control did not correspond to fluorescent spots in either of the root extracts. The glycone of scopoletin, scopolin, was not available for testing. The latter is known to occur in potato tissue in response to infection by viruses and fungi (Clarke, 1973), and may have been present in these extracts.

Discussion

The phenomenon of autofluorescence associated with the hypersensitive response of infected plant tissues has previously been reported in many plant-fungal interactions (Mansfield, Hargreaves & Boyle, 1974; Mayama & Shishiyama, 1976; Kidger & Carver, 1981). The techniques described in this paper have enabled the production of fine root systems of potato that permit direct observation of nematode invasion of intact roots by bright field and fluorescence microscopy. We found fluorescence to be localised at the area of damage caused by PCN in potato roots and the experimental methods enabled the timescale and cellular localisation of fluorescence associated with the plant response to be determined.

These results indicate that the fluorescent response is localised at infection sites and was detected within 3 h of invasion. The early response occurs with both compatible and incompatible interactions but varies in its extent. Because of the short timescale and the differences in the extent of the fluorescence depending on host-parasite compatibility, the response has been termed hypersensitive. Differences in hypersensitivity may

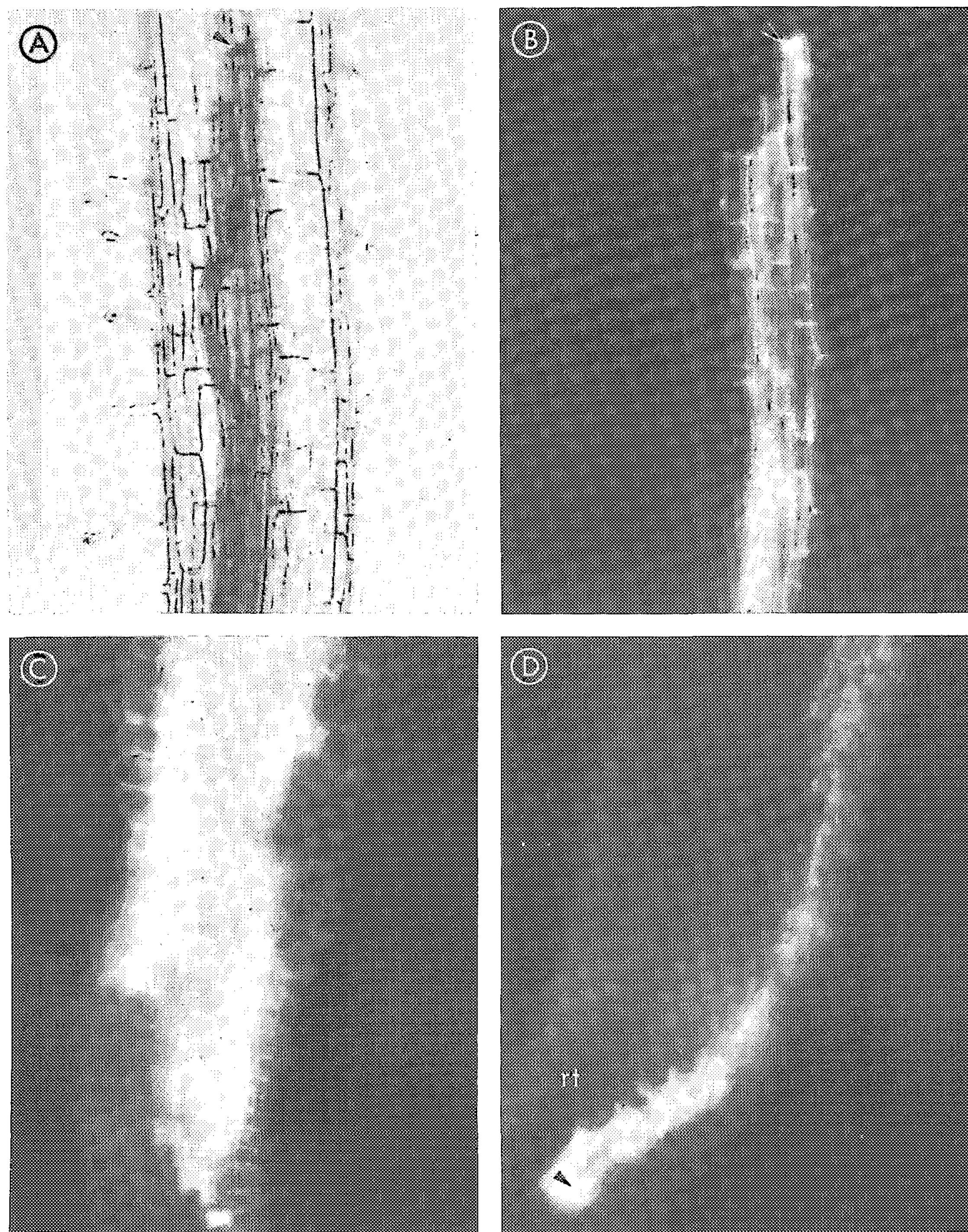

Fig. 2. A & B : Bright field and fluorescence micrographs of *Globodera rostochiensis* tracks through Arran Banner root (24 h); C : Extensive entry point fluorescence of *G. pallida* on 12380/2 (36 h); D : *G. rostochiensis* in the root tip of 12380/2 surrounded by intensive fluorescence (24 h) (× 150).

Arrows indicate anterior end of nematodes; n : nematodes; rt : root tip; s : developing syncytium; h : hours after nematode invasion.

Table 2

Rf values of fluorescent spots detected after t.l.c. of infected and uninfected roots, and phenolic standards.

	Fluorescence in UV light(1)	*Rf (× 100) in BAW*(2)
Unhydrolysed		
Infected	blue-green	25.4, 83.1, 93.0
Uninfected		25.4, 93.0
Chlorogenic acid	blue-green	26.8
Hydrolysed		
Infected	blue	86.4, 95.5
Uninfected		95.5
Caffeic acid	blue	88.1
Scopoletin	bright blue	81.7

(1) 366 nm
(2) BAW = butan-1-ol, acetic acid, water (6 : 1 : 2)

also be related to a wound response (Uritani, 1976; Ryan, 1984) which varies among potato cultivars. However, observed fluorescence following artificial injury to roots with fine glass tubing was more diffuse and shorter lasting than the response to nematode invasion. The initial response appears to be distinct from subsequent fluorescence noted around the developing syncytium at 96 h which is discussed later.

The differences in the extent of the initial response, exhibited by the number of cortical cells affected on either side of the nematode track, indicate that in the incompatible interactions the plant may have been actively responding to a nematode elicitor. It has been suggested that this elicitor might be a component of the nematode cuticle or of secretions or excretions produced by the invading parasite (McClure, Misaghi & Nigh, 1973; Jones, 1975; Rice, Leadbeater & Stone, 1986). The hypersensitive response does not appear to inhibit the subsequent induction of a feeding site but it is not known whether or not the increased initial response in incompatible interactions affects the normal development of the syncytium.

There is a correlation between the accumulation of fluorescent compounds and the distance and direction nematodes moved within the roots and possibly the nematode altered its behaviour as a result of the accumulation of these fluorochromes or their precursors. Differences in track length between the species were observed in the susceptible cultivar Arran Banner but they became more significant in Maris Piper where there was a greater fluorescent response to *G. rostochiensis*. Both species travelled the shortest distance in 12380/2 where fluorescence along the track was greatest. There was a good correlation between extent of fluorescence along the track and the distance the nematodes travelled within the roots. The stimuli that induce PCN to travel a number of body lengths through cortical tissue towards the vascular bundle are not known. It appears to be important, however, that cells within the stele are incorporated into the developing syncytium once the nematode has begun feeding (Jones, 1972). The induction of a syncytium at the correct site is therefore necessary for successful development, and disorientation of the nematodes through the action of chemicals involved in the hypersensitive response may influence this process.

Previously, the fluorescent response following pathogen invasion has been attributed to the presence of polyphenols or their precursors (Mayama & Shishiyama, 1978; Ride, 1983), scopolin or the aglycone scopoletin (Clarke, 1973; Cohen, 1975) or to the presence of the phytoalexins wyerone and wyerone acid (Mansfield, Hargreaves & Boyle, 1974). In the present work, preliminary chromatographic analyses of infected potato root extracts indicated that the fluorescent compounds probably belong to the family of phenylpropanoid plant phenolics (Harborne, 1980) and there was evidence that both chlorogenic and caffeic acids were present. This interpretation was supported by the analysis of fluorescence spectra for roots *in vivo*. More detailed extraction and analytical procedures are required to confirm these results. Chlorogenic acid accumulation has also been reported in resistant tomato roots in response to infection by species of the nematode genera *Meloidogyne* and *Pratylenchus* (Hung & Rohde, 1973) and in alfalfa roots infected by *Hoplolaimus galeatus* (Ahmad & Chen, 1983). Phenols are stored in plants as glycosides and upon infection are oxidised by phenolases and peroxidases, both enzymes being widespread in nature and tending to accumulate in infected tissues (Farkas & Kiraly, 1962). Simple phenols have been shown to influence the mobility of nematodes (Chang & Rohde, 1969) and these may have influenced the behaviour of *G. rostochiensis* in Maris Piper and both species in clone 12380/2. This aspect could be examined further with *in vitro* motility tests using extracts from infected root tissues.

Oxidised polyphenols may also condense to polyquinoid structures such as lignin (Ride, 1983). Previously, histochemical and autofluorescence studies of sectioned tissues have suggested that lignin (Giebel, 1970; Arya, Meenakski & Tiagi, 1985) and callose (Bleve-Zacheo, Melillo & Lamberti, 1982) accumulate in nematode infested roots. Our observation of fluorescence surrounding the developing syncytium in 12380/2 suggests that lignin may accumulate in this region and act as a barrier restricting the flow of nutrients to the parasite. Electron microscopy studies have also confirmed that outer syncytial walls become thickened in PCN infested roots (Rice, Leadbeater & Stone, 1986). In our experiments, this effect was more obvious in clone 12380/2 than in the other resistant cultivar tested, Maris Piper, and may reflect the differences in the genetic basis of resistance of *S. vernei* and H_1 mediated responses (Tur-

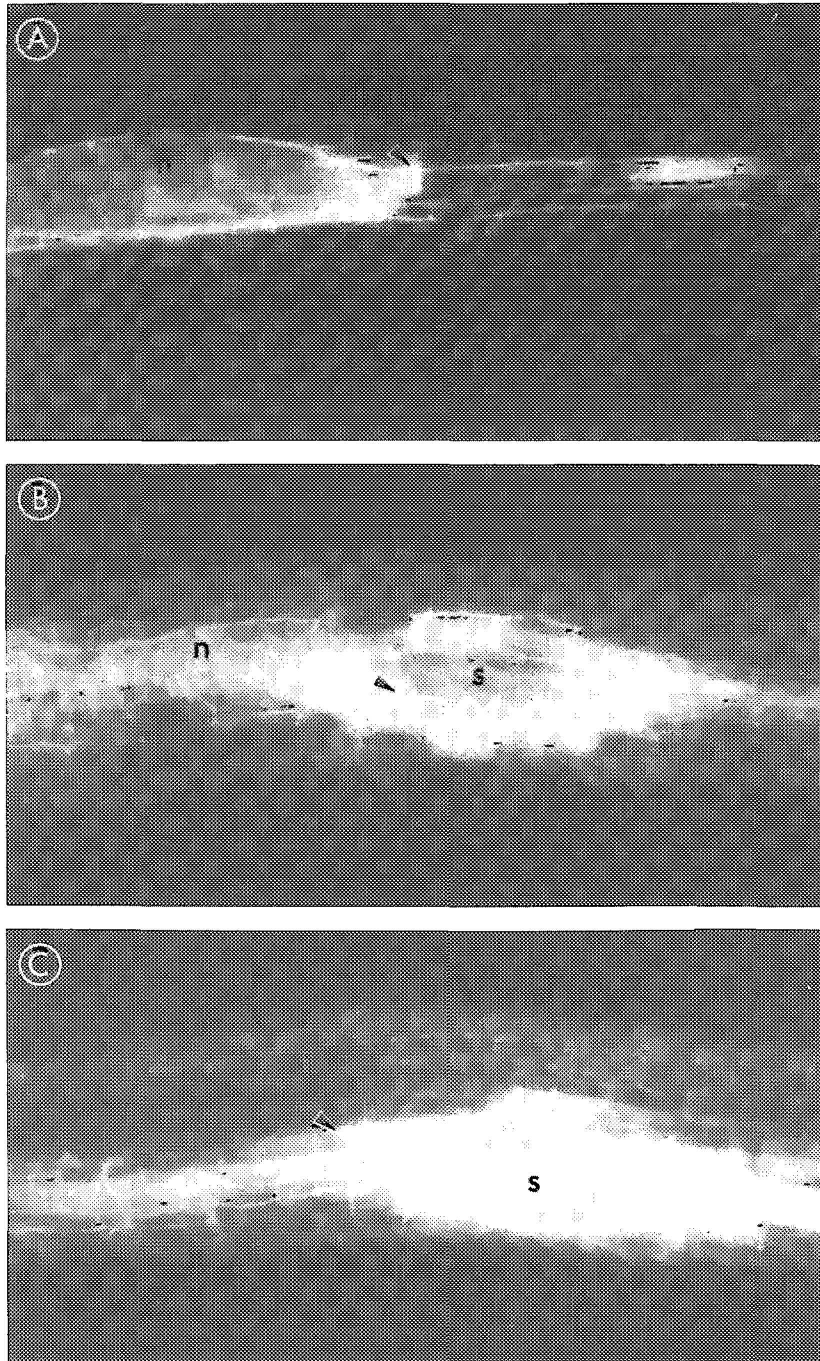

Fig. 3. Fluorescence micrographs. A : Third stage juvenile of *Globodera rostochiensis* developing in roots of Arran Banner (12 d); B & C : *G. rostochiensis* and *G. pallida* (respectively) second stage juveniles showing fluorescence surrounding the feeding site in 12 380/2 (96 h) (× 150).

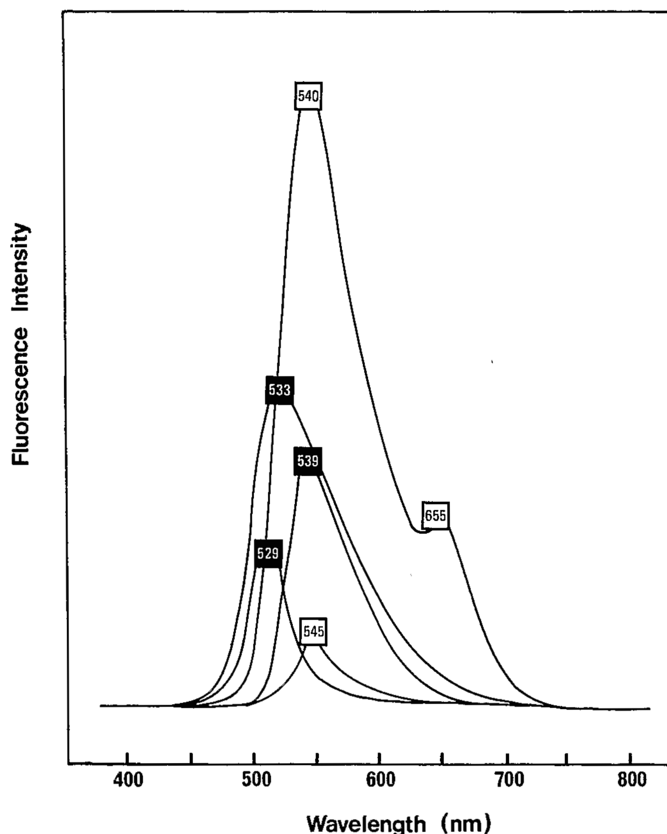

Fig. 4. Fluorescence spectra designated by peaks for emission in nm. Roots 24 h post infection (540 & 655), uninfected roots (545), caffeic acid (539), chlorogenic acid (529) and scopoletin (533).

ner & Stone, 1984). These events occur between three and five days following infection and are likely to be distinct from the initial hypersensitive response. However, the elicitors which induce the early hypersensitive reaction may also be involved in the induction of wound signals (Ryan, 1984) which play a more systemic role and subsequently affect normal syncytial development or modify the plant response to further nematode invasion. Future work will examine these aspects and will also use whole root observations to examine the association of non-fluorescing chemicals with the hypersensitive response.

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